Planta (2002) 215: 177–184
DOI 10.1007/s00425-001-0731-z

ORIGINAL ARTICLE

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# Activity of cell-wall degradation associated with differentiation of isolated mesophyll cells of *Zinnia elegans* into tracheary elements

Received: 3 May 2001 / Accepted: 18 October 2001 / Published online: 8 February 2002


**Abstract** Cell walls were prepared from cultured mesophyll cells of *Zinnia elegans* L. that were transdifferentiating into tracheary elements and incubated in a buffer to undergo autolysis. The rate of autolysis of cell walls was determined by measuring the amount of carbohydrate released from the cell walls into the buffer during incubation. During the course of culture of mesophyll cells, the autolysis rate increased markedly at the time when thickenings of secondary cell walls characteristic of tracheary elements became visible (after 48–72 h of culture), and thereafter the rate remained at a high level. Comparative studies on the autolysis rate of cell walls using various control cultures, in which tracheary element differentiation did not take place, revealed a close relationship between the autolysis rate around the 60th hour of culture and differentiation. Sugar analysis by colorimetric assays and gas chromatography of carbohydrates released from the cell walls detected uronic acid, arabinose, galactose, glucose, xylose, rhamnose, fucose, and mannose. Among these sugars, uronic acid was the most abundant, and accounted for approximately half of the total released sugars. The decrease of acidic polysaccharides in the primary cell walls during tracheary element differentiation was visualized by staining cultured cells with alcian blue at pH 2.5. These results suggest that active degradation of components of primary cell walls, including pectin, is integrated into the program of tracheary element differentiation.

**Keywords** Autolysis · Cell wall · Pectin · Tracheary element · *Zinnia* (cell wall)

**Abbreviations** Ara: arabinose · Ara*f*: arabinofuranose · Fuc: fucose · Gal: galactose · GalA: galacturonic acid · Glc: glucose · GlcA: glucuronic acid · Man: mannose · Rha: rhamnose · TE: tracheary element · Xyl: xylose

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## Introduction

During cytodifferentiation into tracheary elements (TEs), cell walls undergo remarkable structural changes such as localized thickening of secondary cell walls, lignification of secondary walls, and partial degradation of primary walls. Extensive research has been carried out on secondary wall formation and lignin accumulation to elucidate the molecular mechanisms underlying these events. In contrast, the primary wall degradation associated with TE differentiation has received only limited attention. Our current knowledge of this process has come from electron-microscopic observations of developing TEs.

O'Brien (1970) showed by electron microscopy of oat and kidney beans that the primary wall between the bands of thickening secondary wall was digested during TE differentiation, whereas the primary wall beneath the

bands of thickening secondary wall was not. Closer observation of the sequence of TE differentiation in bean indicated the occurrence of primary wall degradation in the presence of a degenerating protoplast (Esau and Charvat 1978). From these observations, it was inferred that the primary wall degradation associated with TE differentiation results from the attack of non-protected walls by hydrolases, which accumulate in developing TEs and are released from the maturing TE after the collapse of the cell membrane. However, O'Brien (1981) presented a contrasting view of this scenario. He interpreted the swelling of primary walls prior to breakdown of the cell membrane as an indication of the initiation of wall degradation by hydrolases, which the cytoplasm of developing TEs secretes actively. Neither of these hypotheses has been tested because of a lack of biochemical and molecular data on the hydrolases responsible for wall degradation.

A typical example of primary wall degradation was reported for lateral walls of developing TEs with an annular pattern of secondary wall thickenings in the elongation zone (O'Brien 1981). Such wall degradation is interpreted to have a role in plasticizing the wall of the dead TE and enabling it to extend passively in association with extension of adjacent living cells. The primary wall degradation occurs, not only at lateral walls, but also at end walls of TEs (Murmanis 1978; O'Brien 1981). The degradation of end walls is apparently related to perforation of vessels. It seems likely that the unlignified region of the future perforation plate at the end wall is hydrolyzed to become finely fibrous and that the pore between TEs is created through the collapse of this hydrolyzed part of the end wall. Thus, primary wall degradation, both at the lateral walls and at the end walls of TEs, is considered to be indispensable for the formation of functional vessels.

Biochemical and molecular biological analyses are absolutely necessary to obtain a better understanding of the TE-associated degradation of primary walls, a process that may be integrated as an essential event in the program of TE differentiation. In vitro culture of mesophyll cells of zinnia (*Zinnia elegans*) provides an experimental system suitable for such analyses. In this system, single cells isolated mechanically from the mesophyll of zinnia can be induced to differentiate into TEs at a relatively high frequency and with good synchrony in a suspension culture (Fukuda and Komamine 1980). Furthermore, observations from electron microscopy of TEs differentiating in this in vitro system demonstrated that primary wall degradation takes place in the same manner as that reported for in vivo TEs (Burgess and Linstead 1984; Nakashima et al. 2000).

Analysis of saccharides released through autohydrolysis of isolated cell walls during incubation is also a very effective method for studying biochemical aspects of wall degradation (Bucheli et al. 1987; Rushing and Huber 1987; Dopico et al. 1989; Dutta et al. 1994). In the present study, we established a similar assay system that could detect the wall-hydrolytic activity by using whole cell walls prepared from cultured zinnia cells differentiating into TEs. This assay system allowed us to measure quantitative changes in the wall-autolysis rate during TE differentiation under the least influence of wall synthesis. Moreover, gas-chromatographic analysis of released sugars was conducted to characterize wall degradation coupled with TE differentiation. On the basis of the data obtained, an outline of wall degradation during TE differentiation is discussed from a biochemical viewpoint.

## Materials and methods

### Plant material

Seeds of *Zinnia elegans* L. cv. Canary Bird (Takii Shubyo, Kyoto, Japan) were sterilized for 10 min in a solution of sodium hypochlorite (0.4%) and germinated on moistened vermiculite. Seedlings were grown under a 14 h light/10 h dark regime at 25 °C.

### Preparation and culture of single mesophyll cells

Mesophyll cells were prepared from the first true leaves of 14-day-old seedlings as described by Fukuda and Komamine (1980). After the leaves had been surface-sterilized in a solution of sodium hypochlorite (approx. 0.15%) and Triton X-100 (0.001%), they were macerated with a blender in a culture medium. Mesophyll cells released from the leaves were filtered through a nylon screen with a pore size of 72 μm, precipitated by centrifugation at 150 *g* for 1 min, and washed with culture medium (Fukuda and Komamine 1980). The isolated cells were resuspended at a cell density of about $8\times10^4$ cells per ml in the culture medium. To induce the formation of tracheary elements, the medium was supplemented with 0.1 mg $l^{-1}$ 1-naphthaleneacetic acid and 0.2 mg $l^{-1}$ benzyladenine (D medium). In control cultures, in which formation of tracheary elements was not induced, the medium was supplemented with 0.1 mg $l^{-1}$ 1-naphthaleneacetic acid and 0.001 mg $l^{-1}$ benzyladenine (C medium). Cultures were maintained in darkness at 27 °C with continuous rotation on a revolving drum (10 rpm).

### Preparation of cell walls

The cultured cells were collected by centrifugation at 1,000 *g* for 5 min or by filtration through a nylon screen (pore size = 10 μm), frozen in liquid nitrogen, and stored at –80 °C. Later, the frozen cells were suspended in 10 vol. of 50 mM Tris-HCl buffer (pH 7.0) and homogenized using an ultrasonic disintegrator (UD-201; Tomy Seiko Co., Tokyo, Japan; output 3, duty 50) for 5 min in an ice bath. The cell wall material was precipitated by centrifugation at 1,000 *g* for 5 min at 4 °C. The pellet was washed three times with 5 mM Tris-HCl buffer (pH 7.0) containing 1% (w/v) Triton X-100 and subsequently three more times with 5 mM Tris-HCl buffer (pH 7.0). Finally, the resultant pellet was used as a cell wall preparation for autolysis experiments. Microscopic observation of this fraction confirmed that it consisted of large fragments of cell walls and negligible amounts of unbroken cells.

### Assay of cell-wall autolysis

The rate of autolysis of the isolated cell walls was measured according to the procedure described by Dutta et al. (1994). The cell walls prepared from approximately $10^6$ cells were resuspended in 20 mM citrate-phosphate buffer (pH 5.5) and distributed into test tubes. Half of the tubes (0 h control) were immediately centrifuged twice at 14,000 *g* for 5 min to remove cell walls from the reaction

mixture. The remaining tubes were incubated for 2 h at 30 °C. After incubation, the cell walls were removed from the reaction mixture by repeating the centrifugation at 14,000 *g* for 5 min. Sugars in the supernatant, released from the cell walls during incubation as a result of autolysis, were quantified and analyzed. The rates of cell-wall autolysis were calculated by subtracting 0 h control values from these values, and are presented in micrograms per $10^6$ cells that were viable at the initiation of culture.

### Determination of sugar content

The amounts of total carbohydrate and uronic acid were determined by the phenol-sulfuric acid method (Dubois et al. 1956) with glucose as a standard, and by the *m*-hydroxybiphenyl assay (Blumenkrantz and Asboe-Hansen 1973) with galacturonic acid as a standard, respectively.

Neutral sugar content was estimated by the anthrone assay according to Dische (1962) with slight modifications as follows. Five hundred microliters of the sample solution was carefully added to a test tube containing 1 ml of anthrone-sulfuric acid solution (2% anthrone in concentrated sulfuric acid) that had been chilled in ice-cold water. These two solutions were mixed thoroughly and then heated in boiling water for 75 s. After cooling in ice-cold water, the $A_{620}$ of the mixture was measured to quantify neutral sugars with glucose as a standard. This modified anthrone assay realized the colorimetric determination of the amounts of pentose and hexose with equal sensitivity.

### Gel-filtration chromatography

Distribution of the molecular weight of autolysis products (sugars released from cell walls during autolysis assay) was analyzed by gel-filtration chromatography on a Bio-Gel P-2 column (10 cm long, 0.5 cm i.d.; Bio-Rad Laboratories, Hercules, Calif., USA). Sugars were eluted from the column with 20 mM citrate-phosphate buffer (pH 5.5) and the total sugar content in each fraction (100 µl) was measured by the phenol-sulfuric acid method.

### Gas-chromatographic analysis of glycosyl-residue and glycosyl-linkage compositions

Glycosyl-residue composition of autolysis products was determined according to the procedure described by Albersheim et al. (1967). After the released sugars were hydrolyzed with 2 M trifluoroacetic acid, sample solutions were desalted by successive ion-exchange chromatographies with Dowex 50 W ×8 (Dow Chemical Co., Midland, Mich., USA) and Amberlite IRA-400 ×8 (Organo Co., Tokyo, Japan) columns. During this procedure, uronic acids as well as buffer salts were removed. The remaining neutral sugars were converted to alditol acetate derivatives, separated by gas chromatography on an SP-2330 column (30 m long, 0.25 mm i.d.; Supelco, Belleford, Pa., USA) at 220 °C, and quantified with a flame-ionization detector.

Glycosyl-linkage composition of autolysis products was determined by a modified version of the Hakomori procedure (Sandford and Conrad 1966). After methylation of the released sugars followed by hydrolysis, partially methylated monosaccharides were converted into alditol acetates. These derivatives were separated by gas chromatography and quantified by a flame-ionization detector in a way similar to that described above. Each derivative was identified by its retention time and mass spectrum.

### Staining of pectic substances with alcian blue

Cultured cells were plasmolyzed by hypertonic treatment with 10% (w/v) of mannitol for 30 min. The cells were then fixed in 35 mM sodium phosphate buffer (pH 7.0) containing 3% formaldehyde and 1.5% glutaraldehyde for 1 h. The fixed cells were soaked for 30 min in 1% alcian blue 8GX (Wako Pure Chemical Industries, Osaka, Japan) that had been dissolved in 3% acetic acid, pH-adjusted to 2.5, and filtered. After washing several times with 10% mannitol, the cells were observed under a light microscope.

## Results

### In vitro assay of cell-wall autolysis

Cell walls were isolated from cells that had been cultured for 60 h in the D medium and had undergone TE differentiation. Incubation of the isolated cell walls in 20 mM citrate-phosphate buffer (pH 5.5) resulted in the release of sugars from the cell walls into the buffer, which could be detected by the phenol-sulfuric acid method after 1 h incubation (Fig. 1). The amount of released sugars continued to increase, although at a slower rate, for five more hours. Because the least amount of sugar was released into the buffer when boiled cell walls were used, sugar release from the isolated cell walls was concluded to depend on the biological activity of the isolated walls to degrade their own polysaccharides. This activity varied, depending on the pH of the buffer used for incubation (Fig. 2). The pH optimum for the autolysis was found to lie between 5.5 and 6.5.

### Changes in the activity of cell-wall degradation during culture

To examine relationships between cell-wall degradation and TE differentiation, cells were cultured in conditions that were either favourable or unfavourable for the induction of TE differentiation, and changes in the rate of cell-wall autolysis were monitored throughout the culture.

In the first experiment, TE differentiation was manipulated by the concentration of cytokinin. One-half of

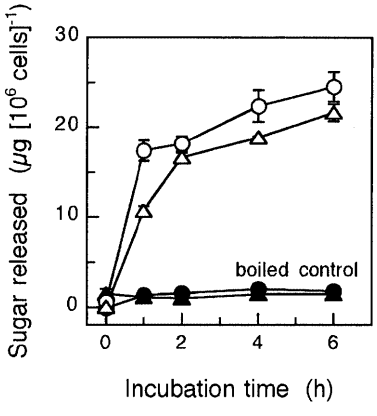


**Fig. 1** Autolysis of isolated cell walls of zinnia (*Zinnia elegans*) incubated in 20 mM citrate-phosphate buffer (pH 5.5) as a function of time. Walls were prepared from cells cultured for 60 h in D medium. Control walls (*closed symbols*) were boiled for 10 min prior to incubation. Total released sugars were quantified by the phenol-sulfuric acid method. This figure shows the results of two independent experiments (*circles* Expt. 1, *triangles* Expt. 2). Assays were performed in duplicate for each experiment. Symbols and bars indicate mean values and SD, respectively

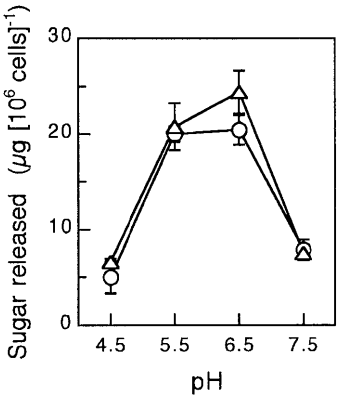


**Fig. 2** pH-dependence of the autolytic activity of isolated zinnia cell walls. Cell walls prepared from cells cultured for 60 h in D medium were incubated in 20 mM citrate-phosphate buffers at pH 4.5, 5.5, 6.5 or 7.5 for 2 h. Total released sugars were quantified by the phenol-sulfuric acid method. This figure shows the results of two independent experiments (*circles* Expt. 1, *triangles* Expt. 2). Assays were performed in duplicate for each experiment. Symbols and bars indicate mean values and SD, respectively

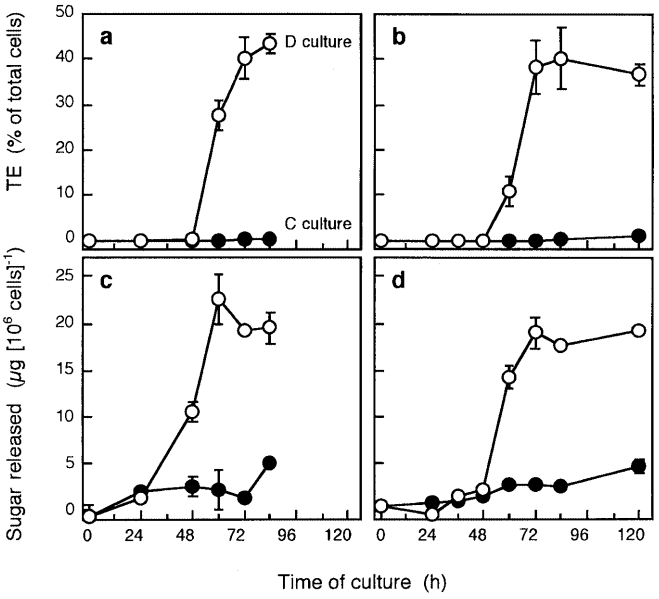


**Fig. 3a–d** Changes in the autolytic activity of zinnia cell walls during culture of isolated mesophyll cells in D medium (*open circles*) or in C medium (*closed circles*). Graphs in this figure show the results of two independent experiments (**a, c** Expt. 1, **b, d** Expt. 2). **a, b** Changes in the proportion of TEs to total cells. TEs were detected by observation of secondary cell wall thickenings under a light microscope. Each symbol represents a mean value of three replicates and bars indicate SD. **c, d** Total sugars released during 2 h incubation of cell walls prepared from cells at various times of culture. Total released sugars were quantified by the phenol-sulfuric acid method. Assays were performed in duplicate. Symbols and bars indicate mean values and SD, respectively

cells were cultured in the D medium for the induction of TE differentiation, and the other half were cultured in the C medium in which benzyladenine was reduced to a concentration insufficient for induction of TE differentiation. In the D culture, TEs became visible due to localized thickening of secondary walls after 48 h, and the proportion of TEs to total cells increased rapidly up to approximately 40% during the subsequent 24 h (Fig. 3a, b). The increased number of TEs in the D culture was accompanied by an elevation of the activity of cell-wall degradation, which was detected slightly ahead of the appearance of visible TEs (Fig. 3c, d). By contrast, the C culture induced neither TE differentiation nor activation of cell-wall degradation (Fig. 3).

Second, TE differentiation was manipulated by treatment with nicotinamide, which has been demonstrated to block differentiation without inhibiting cell division in the present culture system (Sugiyama and Komamine 1987). As shown in Fig. 4a, b administration of nicotinamide at concentrations of 5 mM and 20 mM resulted in partial and complete prevention of TE formation, respectively. In the former case, the final proportion of TEs to total cells was reduced to 12–22%, whereas the timing of the differentiation was not affected. Nicotinamide substantially suppressed the increase in the activity of cell-wall degradation but did not severely inhibit the later activity (Fig. 4c, d). Levels of degradation activity after 60 h of culture were nearly proportional to the frequencies of TE differentiation.

## Molecular-weight distribution of the products of cell-wall autolysis

Autolysis products of cell walls prepared from cells that had been cultured for 60 or 96 h in the D medium were fractionated by gel-filtration chromatography to estimate their molecular-weight distribution. In both 60-h and 96-h samples, approximately 30% of total sugars was eluted in the low-molecular-weight fractions corresponding to monomeric to pentameric saccharides (Fig. 5).

## Glycosyl-residue and glycosyl-linkage compositions of the products of cell-wall autolysis

To gain insight into the kinds of wall polysaccharides that are degraded during TE differentiation, autolysis products of cell walls prepared from cells that were cultured for 60 h in the D medium were subjected to colorimetric and gas-chromatographic analyses.

The contents of neutral sugars and uronic acids in the autolysis product were estimated by the anthrone assay and the *m*-hydroxybiphenyl assay to be (mean ± SD) $28.54 \pm 0.41$ and $27.68 \pm 0.83$ μg per $10^6$ cells, respectively. The sum of these values was much larger than the amount of total sugars determined by the phenol-sulfuric acid method ($20.32 \pm 0.81$ μg). This apparent discrepancy was attributed to the relatively low sensitivity of the phenol-sulfuric acid method to several sugar species, including uronic acids and deoxy sugars. Analysis of the uronic acids in the autolysis product by gas chromatography following their conversion to trimethylsilyl derivatives indicated that 82% of the

uronic acid residues were galacturonic acid (GalA) and that the remainder was glucuronic acid (GlcA).

Glycosyl-residue composition of the fraction of neutral carbohydrates prepared from the autolysis product was determined by gas chromatography after neutral sugars were converted to alditol acetate derivatives. As shown in Table 1, seven sugars [arabinose (Ara), galactose (Gal), glucose (Glc), rhamnose (Rha), xylose (Xyl), fucose (Fuc), mannose (Man)] were detected as glycosyl residues in this fraction. Among these sugar species, Ara was most abundant and accounted for 38% of neutral sugars released from cell walls. Glycosyl-linkage composition of the autolysis product, determined by methylation analysis, indicated that the autolysis product contained relatively high percentages of arabinofuranose (5-Ara*f*, T-Ara*f*) and T-Gal (Table 2).

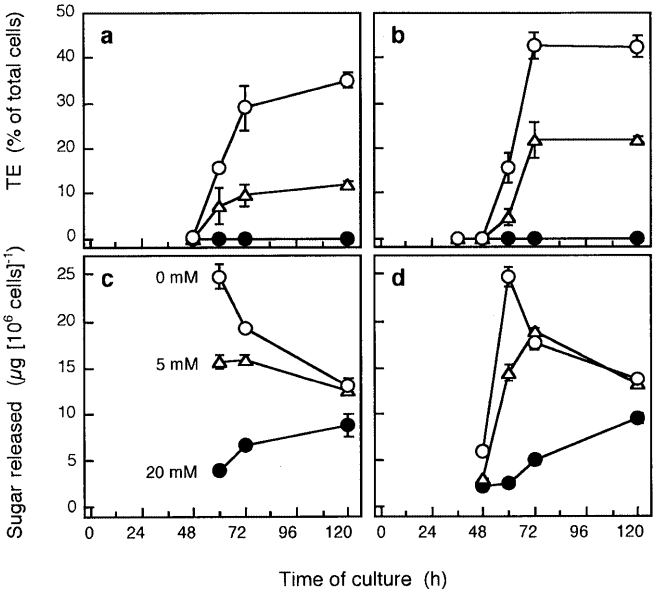


**Fig. 4a–d** Changes in the autolytic activity of zinnia cell walls during culture of isolated mesophyll cells in D medium that contained no nicotinamide (*open circles*), 5 mM nicotinamide (*open triangles*), or 20 mM nicotinamide (*closed circles*). Graphs in this figure show the results of two independent experiments (**a, c** Expt 1, **b, d** Expt. 2). **a, b** Changes in the proportion of TEs. Each symbol represents a mean value of three replicates and bars indicate SD. **c, d** Total sugars released during 2 h incubation of cell walls prepared from cells at various times of culture. Total released sugars were quantified by the phenol-sulfuric acid method. Assays were performed in duplicate. Symbols and bars indicate mean values and SD, respectively

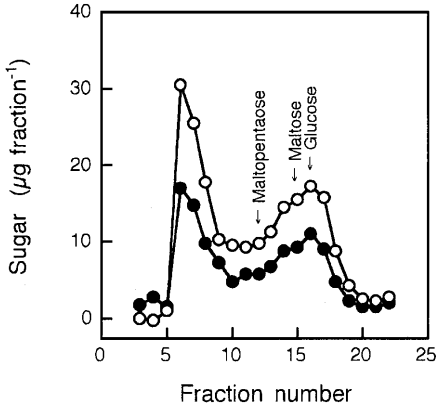


**Fig. 5** Chromatography, on a Bio-Gel P-2 column, of sugars released from zinnia cell walls after autolysis reaction. The cell walls were isolated from cells cultured for 60 h (*open circles*) or 96 h (*closed circles*) in D medium. Sugars were eluted with 20 mM citrate-phosphate buffer (pH 5.5) and the total sugar content in each fraction was measured by the phenol-sulfuric acid method. Glucose, maltose, and maltopentaose were used as standards to calibrate molecular weight. Assays were performed in duplicate

## Alcian-blue staining of differentiating TEs

To visualize changes in the content of pectic substances in cell walls during TE differentiation, immature and mature TEs and non-TE cells were stained in the alcian blue solution (pH 2.5), in which pectic substances containing large amounts of carboxyl groups were expected to assume blue coloration. While cell walls of non-TE cells and immature TEs turned blue, walls of mature TEs did not (Fig. 6). This result implies that pectic sub-

**Table 1** Glycosyl-residue composition of neutral carbohydrates released from zinnia (*Zinnia elegans*) cell walls through their autolysis. The cell walls were prepared from cells that were cultured in three replicates for 60 h in the D medium. Values are means ± SD

| Glycosyl-residue | Mol% |
|---|---|
| Rha | 11.2 ± 0.2 |
| Fuc | 3.1 ± 0.7 |
| Ara | 38.0 ± 0.7 |
| Xyl | 10.1 ± 0.6 |
| Man | 1.8 ± 0.1 |
| Gal | 18.5 ± 0.2 |
| Glc | 17.2 ± 0.9 |
| Total | 100 |

**Table 2** Glycosyl-linkage composition of neutral carbohydrates released from zinnia cell walls through their autolysis. The cell walls were prepared from cells that were cultured in three replicates for 60 h in D medium. Values are means ± SD

| Sugar | Linkage | Mol% |
|---|---|---|
| Rha | T- | 2.4 ± 0.6 |
| | 2- | 8.5 ± 2.7 |
| | 3- | 2.2 ± 0.3 |
| | 2,4- | 8.1 ± 0.7 |
| Fuc | T- | 2.3 ± 0.0 |
| Ara*f* | T- | 11.2 ± 1.6 |
| | 5- | 17.2 ± 2.9 |
| Xyl | T- | 6.4 ± 1.7 |
| | 4- | 7.7 ± 0.8 |
| Gal | T- | 9.6 ± 1.3 |
| | 4- | 5.5 ± 0.3 |
| | 2,4- | 3.3 ± 0.6 |
| Glc | T- | 1.1 ± 0.5 |
| | 4- | 6.8 ± 0.5 |
| | 4,6- | 8.0 ± 0.8 |
| Total | | 100 |

stances were markedly reduced during maturation of differentiating TEs.

## Discussion

In the studies described in this paper, we successfully applied the in vitro culture system of zinnia mesophyll cells and the cell-wall autolysis assay to permit biochemical characterization of the degradation of cell walls associated with TE differentiation. When isolated mesophyll cells were induced to differentiate into TEs in a culture in the D medium, the rate of cell-wall autolysis was elevated after 48 h and reached a maximum during the subsequent 24 h, which was maintained for the following 48 h (Fig. 3). This presents a marked contrast to the very low rate of autolysis throughout culture in the C medium, where few cells differentiated into TEs. The relationship between TE differentiation and the activity of cell-wall degradation was further examined by treating cells with nicotinamide. Administration of nicotinamide to the D cultures, which reduced the frequency of TE differentiation without changing its timing, strongly suppressed the increase in the rate of cell-wall autolysis around the 60th hour of culture, with much smaller effects on the autolysis rate at later stages (Fig. 4). These results clearly indicate that the increase in the activity of cell-wall degradation around the 60th hour was closely linked with TE differentiation, whereas the high activity of degradation after that time was not directly attributable to TE differentiation.

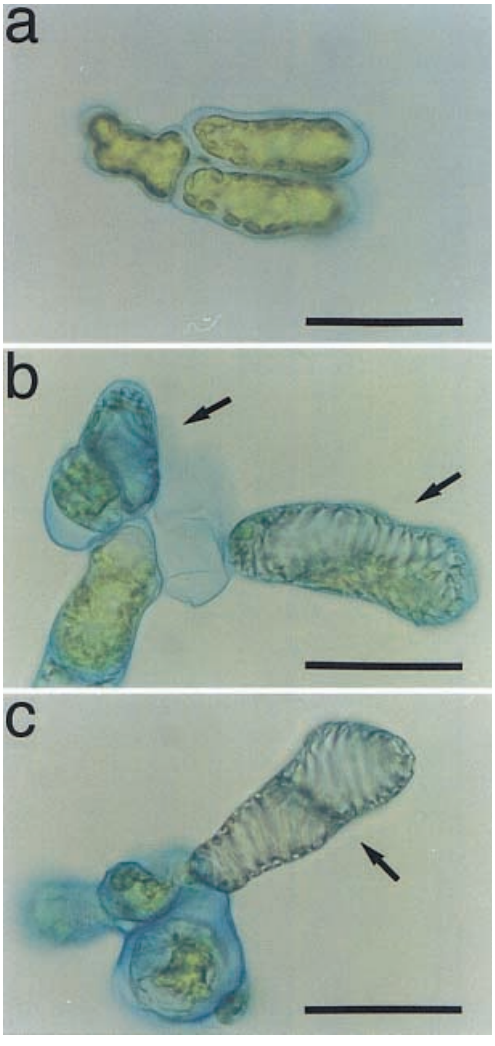


**Fig. 6** Staining of cells cultured for 0 h (**a**), 72 h (**b**), and 96 h (**c**) in D medium with alcian blue at pH 2.5. *Arrows* indicate immature (**b**) or maturing (**c**) TEs. Bars = 50 μm

Cell-wall materials such as xylan are deposited to form secondary wall thickenings in TEs at the 60th hour of culture in the zinnia system (Ingold et al. 1988). Thus, the activation of cell-wall degradation around this time, which was found to be coupled to TE differentiation, suggests that cell-wall degradation takes place concurrently with secondary wall thickening in developing TEs. However, this argument could be weakened because it is based on the average data for cell populations that contained TEs with some variability in their stage of development. For the same reason, it is impossible at present to determine whether the initiation of cell-wall degradation precedes the breakdown of the tonoplast, which triggers degeneration of intracellular components in maturing TEs (Obara et al. 2001). In future studies, the temporal sequence of events involved in TE differentiation, including cell-wall degradation, should be examined for individual cells differentiating into TEs.

Gel-filtration chromatography of the carbohydrates released from cell walls through their autolysis detected a large amount of sugars in the low-molecular-weight fractions (approx. 50% of total sugars), corresponding to monomeric to pentameric saccharides (Fig. 5). This result indicates that most of the carbohydrate molecules in the autolysis product had very short glycosyl chains, suggesting that cell-wall polysaccharides suffer extensive degradation into small fragments during TE differentiation.

Glycosyl-residue and glycosyl-linkage compositions of the autolysis product are very informative for determining which polysaccharides are degraded in the cell wall during TE differentiation. Colorimetric analysis of sugars released from the 60-h cell walls through their autolysis showed that the autolysis product contained nearly equal amounts of neutral sugars and uronic acids. When the neutral sugar fraction was subjected to gas-chromatographic analysis, nearly all neutral monosaccharides that are known as components of the cell wall of dicots were detected (Table 1). The most abundant of these was Ara, which accounted for 38% of the neutral sugars and approximately 20% of the total sugars released. In the uronic acid fraction, GalA predominated and accounted for about 40% of the total released sugars. Thus, the most common sugar species in the total autolysis product was GalA, not Ara.

In the light of our knowledge that GalA exists in the pectin fraction of the cell wall as a component of homogalacturonan and rhamnogalacturonan I (Carpita and Gibeaut 1993), the dominance of GalA in the autolysis product represents apparently active degradation of pectic substances during TE differentiation,. Since rhamnogalacturonan I consists of a heteropolymer backbone of repeating (1→2)α-L-rhamnosyl-(1→4)α-D-GalA disaccharide units, degradation derivatives of this polysaccharide should contain equal amounts of GalA and Rha residues. In our data, Rha was detected in the autolysis product, but it was estimated to be less than one-sixth of the amount of GalA. Accordingly, it can be reasonably inferred that only a limited part of the detected GalA residues was derived from rhamnogalacturonan I, whereas the majority was from homogalacturonan.

Ara and Gal comprised a major part of the neutral sugar fraction in the autolysis product. In the linkage analysis, T-Gal, 4-Gal, 2,4-Gal, T-Ara*f*, and 5-Ara*f* were detected with respect to these two sugars (Table 2). The absence of 3,4-Gal and 3,6-Gal in the linkage composition suggested that Gal and Ara were derived from neither pectic arabinogalactan (type I), which comprises the main chain of (1→4)β-D-galactan and Ara residues combined with the main chain at the O-3 position of Gal residues, nor arabino-3,6-galactan (type II), which comprises the main chain of (1→3)β-D-galactan and Ara or Gal residues combined with the main chain at the O-6 position of Gal residues (Carpita and Gibeaut 1993). Instead, in view of the manner in which they are linked, the origins of these sugars are likely to be (1→4)β-D-galactan, and (1→5)α-L-arabinan.

Glucose ranked next to Ara and Gal in the glycosyl-residue composition of the autolysis product (Table 1). Linkage analysis detected 4,6-Glc in addition to T-Glc and 4-Glc (Table 2), which suggests that xyloglucan degradation played some part in the Glc release from cell walls. Xyloglucan degradation can also explain the release of Xyl residues that were detected as T-Xyl in the linkage analysis. On the other hand, linkage analysis of Xyl indicated that the content of 4-Xyl was similar to that of T-Xyl in the autolysis product (Table 2). This manner of Xyl linkage attributes the release of Xyl residues to the degradation of (1→4)β-D-xylan in addition to xyloglucan. Although it is well known that the secondary walls of TEs accumulate xylan, Northcote et al. (1989) reported the existence of xylan in the primary walls as well as the secondary walls. Taking this finding together with observations from electron microscopy that showed selective breakdown of primary walls during TE differentiation (e.g. Burgess and Linstead 1984), a considerable amount of Xyl is supposed to have come from xylan in the primary walls.

When compared, the data in Table 1 and Table 2 for each sugar species showed some discrepancies. For example, the proportion of Rha to total neutral sugars is 11.2% in Table 1, but 21.2% in Table 2. Such discrepancies may be explained by the alkali-lability of monosaccharides. Monosaccharides are more labile in the highly alkaline solution used in the methylation procedure than are sugars of oligo- or polysaccharides, and this is known to cause a selective loss of monosaccharides in the glycosyl-linkage analysis. As indicated by gel-filtration chromatography (Fig. 5), the autolysis product contained a considerable amount of monosaccharides. Assuming that these monosaccharides were different in glycosyl-residue composition from the whole autolysis product, the apparent discrepancies between the data of glycosyl-residue composition and glycosyl-linkage composition are not surprising.

In summary, from the gas-chromatographic analyses of the sugars released from the isolated cell walls through their autolysis, it can be speculated that a wide range of polysaccharides in the cell wall, including homogalacturonan, rhamnogalacturonan I, arabinan, galactan, xyloglucan, and xylan, are degraded into small carbohydrate molecules in association with TE differentiation. In particular, the degradation of pectic polysaccharides, homogalacturonan and rhamnogalacturonan, appeared to be remarkable during TE differentiation. Using the same culture system as employed in the present research, Stacey et al. (1995) showed the secretion of pectic polysaccharides into the culture medium from the cell walls during TE differentiation. Our results suggest active degradation of pectic substances, which is in agreement with this report.

We further examined pectin degradation in situ during TE differentiation by staining cells at pH 2.5 with alcian blue, which interacts electrostatically with carboxyl groups of acidic polysaccharides at this pH (Thornton et al. 1996). As a result, primary cell walls of undifferentiated cells and immature TEs at the initial stage of differentiation were stained blue, whereas cell walls of mature TEs were not stained (Fig. 6). This staining pattern, together with the above-mentioned results, leads to the conclusion that primary cell walls of developing TEs lose pectic substances through their active degradation.

Recently, Domingo et al. (1998) isolated a cDNA clone for the gene (*ZePel*) encoding pectate lyase, a pectin-degrading enzyme, from zinnia. They investigated expression patterns of *ZePel* mRNA and found a marked increase in the mRNA level in cultured zinnia cells induced to differentiate into TEs. Pectate lyase activity was also shown to be elevated markedly in the TE-inductive cultures. These results seem to assign pectin degradation during TE differentiation to the activity of pectate lyase. Nonetheless, we speculate that pectate lyase does not play a critical role in pectin degradation in developing TEs for two reasons. Firstly, like microbial pectate lyases, the pectate lyase encoded by *ZePel* prefers an alkaline pH and has only slight activity at the acidic to neutral pH at which cell-wall autolysis is most active. Secondly, *ZePel* expression and pectate lyase activity are induced in response to auxin rather than in association with TE differentiation. Another type of

pectin-degrading enzyme, polygalacturonase, may have an essential part in pectin degradation during TE differentiation.

**Acknowledgements** This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (No.10219201, No.10182101), and from the Japan Society for the Promotion of Science (No.10304063, JSPS-RFTF96L00605).

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